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<b>(21) International Application Number:</b> PCT/IB95/00445 <b>(22) International Filing Date:</b> 7 June 1995 (07.06.95)  <b>(71) Applicant (for all designated States except US):</b> PFIZER INC. [US/US]; 235 East 42nd Street, New York, NY 10017 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> EVANS, Nigel, A. [GB/US]; 34 Willow Lane, East Lyme, CT 06333 (US). FINDLY, R., Craig [US/US]; 14 Warner Place, Wethers- field, CT 06109 (US). WEBER, Frederick, H. [US/US]; 159 Phoenix Court, Terre Haute, IN 47803 (US).  <b>(74) Agents:</b> SPIEGEL, Allen, J. et al.; Pfizer Inc., 235 East 42nd Street, New York, NY 10017 (US).		<b>(81) Designated States:</b> CA, FI, JP, MX, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> IN OVO VACCINATION AGAINST COCCIDIOSIS  <b>(57) Abstract</b>  The invention relates to a method of vaccinating a domesticated bird against coccidiosis comprising administering <i>in ovo</i> an effective immunizing dose of live <i>Eimeria</i> sporocysts or oocysts, or a mixture thereof.		

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IN OVO VACCINATION AGAINST COCCIDIOSISBackground of the Invention

The present invention relates to a method of vaccinating domesticated birds against coccidiosis. In particular, the invention relates to the *in ovo* administration of live *Eimeria* spp sporocysts or oocysts, or mixtures thereof, into the developing  
10 eggs of domesticated birds in order to immunize the hatched chicks against coccidiosis.

Coccidiosis is an enteric disease of domesticated birds caused by infection with intracellular protozoan parasites of the genus *Eimeria*. Coccidiosis is the most economically devastating parasitic disease of domesticated birds. It is estimated that  
15 anticoccidial medications and losses due to coccidiosis cost the poultry industry hundreds of millions of dollars every year.

Various attempts to vaccinate domesticated birds against coccidiosis have been reported since the early 1950's. Current vaccination methods include administering live *Eimeria* oocysts to birds through feed or water. These methods,  
20 however, are inconvenient and inefficient because not all birds get the intended oocyst dose and many are either unprotected by the vaccine or receive a pathogenic infection.

In J. M. Sharma and B. R. Burmester, Avian Dis. 26: 134-149, 1981, the authors reported that chickens vaccinated *in ovo* with herpesvirus of turkey  
25 developed immunity against subsequent challenge with Marek's disease virus. In European patent publication no. 291173, an immunization process is referred to wherein a nonreplicating immunogen is administered *in ovo*. The immunogens specifically referred to in the European patent are a genetically engineered *Eimeria* antigen and an *Eimeria* oocyst extract. The European patent specifically excludes  
30 live parasite stages such as those used in the vaccination method claimed herein.

The present vaccination method involves *in ovo* administration of live *Eimeria* sporocysts or oocysts, or a mixture thereof, into the developing eggs of domesticated birds. The available literature suggests that such a vaccination method would be ineffective *in ovo* and should be applied post-hatch. In T.K. Jeffers and  
35 G.E. Wagenbach, J. Parasit. 56(4): 656-662, 1970, the authors reported that *in ovo* injection of *E. tenella* sporozoites on day 10 of incubation provided no significant immunological protection against subsequent challenge with *E. tenella* oocysts. In

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fact, they reported that chicks that received no treatment had a greater survival rate against subsequent challenge with *E. tenella* oocysts than chicks that had been treated *in ovo* with sporozoites. In K.L. Watkins et al., Proc. VI th. International Coccidiosis Conf., Abstract E1-2, Ontario, Canada, 1993, the authors described *in* 5 *ovo* inoculation with live *E. maxima* sporocysts and sporulated oocysts, although they did not indicate when the inoculation was done during incubation of the egg. Watkins et al. concluded that their study provided no evidence that *in ovo* exposure protects against subsequent coccidial challenge with *E. maxima* oocysts 10 days post-hatch. They also concluded that significant immunological protection is 10 provided if inoculation is done soon after hatch rather than *in ovo*. Contrary to this teaching, the *in ovo* vaccination method of the present invention provides unexpected immunity that protects the hatched birds against subsequent coccidial challenge.

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#### Summary of the Invention

The present invention, also referred to herein as the "present vaccination method", relates to a method of vaccinating a domesticated bird against coccidiosis comprising administering *in ovo*, during the final quarter of incubation, an effective immunizing dose of live *Eimeria* sporocysts or oocysts, or a mixture thereof.

20

The term "domesticated bird(s)", as used herein, unless otherwise indicated, includes chickens, turkeys, ducks, game birds (including, but not limited to, quail, pheasants, and geese) and ratites (including, but not limited to, ostrich).

The term "*in ovo*", as used herein, unless otherwise indicated, means into a domesticated bird egg containing a live, developing embryo.

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The term "administering *in ovo*" or "*in ovo* administration", as used herein, unless otherwise indicated, means administering the vaccine described herein to a domesticated bird egg containing a live, developing embryo by any means of penetrating the shell of the egg and introducing the vaccine. Such means of administration include, but are not limited to, injection of the vaccine.

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The term "final quarter of incubation", as used herein, unless otherwise indicated, means the final quarter of incubation of a developing egg of a domesticated bird.

The term "*Eimeria*", as used herein, unless otherwise indicated, means one or more species of the genus *Eimeria* that infect domesticated birds. Such *Eimeria* species include those that are found in chicken, including *E. tenella*, *E. acervulina*, *E. maxima*, *E. necatrix*, *E. mitis*, *E. praecox*, and *E. brunetti*, and also those that are found in turkeys, including *E. meleagrimitis*, *E. adenoeides*, *E. gallopavonis*, *E. dispersa*, *E. meleagris*, *E. innocua*, and *E. subrotunda*, and also *Eimeria* species that infect other domesticated birds as defined above. The term "*Eimeria*" also includes all strains of the foregoing species of *Eimeria*, including, but not limited to, precocious strains, and attenuated strains, which includes strains that have been irradiated, or otherwise treated, so that they fail to complete development. The term *Eimeria* also includes any newly-discovered strains or species of *Eimeria* that infect domesticated birds as defined above.

The term "sporocysts", as used herein, unless otherwise indicated, means live *Eimeria* sporocysts.

The term "oocysts", as used herein, unless otherwise indicated, means live *Eimeria* sporulated oocysts or a mixture of sporulated and unsporulated oocysts.

The term "effective immunizing dose", as used herein, unless otherwise indicated, means a number of sporocysts or oocysts, or, when mixed, a number of sporocysts and oocysts, sufficient to provide immunological protection in the hatched birds that is greater than the inherent immunity of non-immunized birds. As used herein, the terms "immunize" and "vaccinate" are synonymous and are used interchangeably.

A preferred dose to be administered in accord with the method of the invention comprises  $10^2$  to  $10^8$  sporocysts or oocysts, or a mixture thereof wherein the total number of said sporocysts and oocysts ranges from  $10^2$  to  $10^8$ .

A more preferred dose comprises  $10^2$  to  $10^5$  sporocysts or oocysts, or a mixture thereof wherein the total number of said sporocysts and oocysts ranges from  $10^2$  to  $10^5$ .

Another preferred dose comprises  $10^5$  to  $10^7$  sporocysts or oocysts, or a mixture thereof wherein the total number of said sporocysts and oocysts ranges from  $10^5$  to  $10^7$ .

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Another preferred dose comprises  $10^4$  to  $10^6$  sporocysts or oocysts, or a mixture thereof wherein the total number of said sporocysts and oocysts ranges from  $10^4$  to  $10^6$ .

Another preferred dose comprises  $10^3$  to  $10^6$  sporocysts or oocysts, or a mixture thereof wherein the total number of said sporocysts and oocysts ranges from  $10^3$  to  $10^6$ .

A preferred domesticated bird to be vaccinated in accord with the method of the invention is a chicken.

A preferred dose to be administered *in ovo* to chicken eggs comprises sporocysts or oocysts, or a mixture thereof, of two or more species of *Eimeria* selected from the group consisting of *E. tenella*, *E. acervulina*, *E. maxima*, *E. necatrix*, *E. mitis*, *E. praecox*, and *E. brunetti*.

Another preferred domesticated bird to be vaccinated in accord with the method of the invention is a turkey.

A preferred dose to be administered *in ovo* to turkey eggs comprises sporocysts or oocysts, or a mixture thereof, of two or more species of *Eimeria* selected from the group consisting of *E. meleagritidis*, *E. adenoides*, *E. gallopavonis*, *E. dispersa*, *E. meleagridis*, *E. innocua*, and *E. subrotunda*.

Other preferred domesticated birds to be vaccinated in accord with the method of the invention are game birds, ducks and ratites.

The method of the invention further comprises, in combination with the present vaccination method, administering *in ovo* an immune stimulant at any time during incubation.

A preferred method of administering the immune stimulant is simultaneously with the *in ovo* administration of a dose of sporocysts or oocysts, or mixture of said sporocysts and oocysts, during the final quarter of incubation.

#### Detailed Description of the Invention

The present vaccination method involves the *in ovo* administration, during the final quarter of incubation, of live *Eimeria* sporocysts or oocysts, or a mixture thereof, into domesticated birds' eggs. In the case of chickens, *in ovo* administration is preferably done on days 15-20 of incubation, and most preferably on day 18 of

incubation. In the case of turkeys, *in ovo* administration is preferably done on days 21-26 of incubation.

The present vaccination method can be performed using any suitable *in ovo* administration method. Preferably, the present vaccine is administered via injection.

5 According to one method of injection, a hole is made in the egg shell at the large end of the egg using an 18 gauge needle to expose the egg's air cell. A 1.0-1.5 inch 22 gauge needle attached to a syringe of appropriate size (1-3 ml) can be inserted through the hole and through the membrane of the air cell. An appropriate number of sporocysts or oocysts, or, when mixed, an appropriate number of sporocysts and

10 oocysts, are suspended in a suitable liquid carrier, for instance 10-500 $\mu$ l of phosphate-buffered saline, and then injected into the egg. The appropriate volume will depend on the size of the egg being treated, with ostrich eggs obviously being capable of taking more volume than chicken eggs. The site of injection can be within any region of the egg. Preferably, injection is done axially through the center of the

15 large end of the egg into the amnion.

Alternatively, an automated egg injection system can be used in the present vaccination method. Such systems are described in U.S. Patent nos. 4,681,063, 4,040,388, 4,469,047, and 4,593,646, which are herein incorporated by reference. Other appropriate methods of injection are known to those skilled in the art.

20 Oocysts to be used in accord with the present vaccination method can be prepared by any of several methods known to those skilled in the art. Such methods include those described in J.F. Ryley et al., Parasitology 73:311-326, 1976 and P.L. Long et al., Folia Veterinaria Latina VI#3, 201-217, 1976, which are herein incorporated by reference. According to one method, commercial broiler chickens,

25 approximately 2 weeks old, are infected with the *Eimeria* species of interest by oral gavage of an appropriate dose of sporulated oocysts. For example, a typical dose used for *E. tenella* is 200,000 sporulated oocysts/bird. Well known procedures for collection and purification of oocysts from infected birds are then followed. For most species of *Eimeria*, feces are collected from infected birds 5-7 days post-infection,

30 blended and filtered to remove debris, then centrifuged at a speed sufficient to pellet the remaining fecal material. For *E. tenella*, a similar procedure is used except that cecal cores are taken at 6 days post-infection. The pellet is resuspended in a saturated salt solution, in which the oocysts float and most of the contaminating

debris can be removed by centrifugation. The oocyst suspension is then diluted to lower the salt concentration. The oocysts are washed repeatedly to remove the salt and resuspended in potassium dichromate solution (2.5% w/v). The oocyst suspension is incubated at 29°C with shaking (e.g., 140 rpm) for approximately 72  
5 hours to induce sporulation of the oocysts. Alternatively, the oocysts can be treated with sodium hypochlorite and then sporulated. The number of sporulated oocysts/ml is determined by direct count using a hemocytometer, and the culture is stored, preferably under refrigeration until needed. The oocysts can be used in accord with the present vaccination method, preferably in a dose comprising from  $10^2$  to  $10^8$   
10 oocysts per egg. More preferably, the dose comprises from  $10^2$  to  $10^5$  oocysts per egg.

To prepare sporocysts, the potassium dichromate is removed from the oocyst suspension described above by repeated washing of the oocysts, which involves collection of oocysts by centrifugation and resuspending in deionized or distilled  
15 water. When the dichromate has been removed as judged by the lack of yellowish-orange coloration, the oocyst suspension is mixed with an equal volume of sodium hypochlorite (bleach) and incubated at room temperature for 15 minutes. The bleach is then removed by repeated washings, and the oocysts are resuspended in physiological saline or deionized water. Oocysts can be broken to release sporocysts  
20 using a variety of known techniques. For example, oocysts can be broken to release sporocysts by mixing the oocysts with glass beads of 1-4 mm diameter and shaking by hand, vortex mixer, or shaking incubator, or using a hand-held homogenizer. Unbroken oocysts and oocysts walls can be separated from the released sporocysts by differential centrifugation in 50% Percoll® (sold by Pharmacia Biotech) or 1 M  
25 sucrose as described in Dulski et al., Avian Diseases, 32: 235-239, 1988. The sporocysts can be used in the present vaccination method either mixed with or separated from the unbroken oocysts and oocysts walls. Preferably, the dose of sporocysts is separated from the oocysts and oocysts walls. A preferred dose of sporocysts comprises from  $10^2$  to  $10^8$  sporocysts per egg. More preferably, the  
30 dose comprises from  $10^2$  to  $10^5$  sporocysts per egg.

The sporocysts or oocysts, or mixture thereof, can be injected *in ovo* in any physiologically suitable medium. Preferably, they are suspended in physiologically balanced saline such as phosphate buffered saline. The selected medium can



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optionally include one or more suspending agents including physiologically suitable gels, gelatins, hydrosols, cellulose, or polysaccharide gums.

Preferably, in the present vaccination method, sporocysts or oocysts, or a mixture thereof, of two or more *Eimeria* species are injected *in ovo* at the same time.

- 5 In accord with the present vaccination method, sporocysts or oocysts, or a mixture thereof, of all identified species of *Eimeria* that infect a specific domesticated bird, such as chicken, can be injected *in ovo* at the same time, or in series, at appropriate doses to provide immunological protection against all species.

- 10 Immune stimulants can be used in conjunction with the present vaccination method. Immune stimulants that can be used in the present vaccination method include, but are not limited to, cytokines, growth factors, chemokines, supernatants from cell cultures of lymphocytes, monocytes, or cells from lymphoid organs, cell preparations or cell extracts (e.g. fixed *Staphylococcus aureus* or lipopolysaccharide preparations), mitogens, or adjuvants, including low molecular weight  
15 pharmaceuticals. Immune stimulants can be administered *in ovo* at any time during incubation. Preferably, immune stimulants are administered *in ovo* in the medium containing the dose of *Eimeria* sporocysts or oocysts, or mixture thereof.

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We claim:

1. A method of vaccinating a domesticated bird against coccidiosis comprising administering *in ovo*, during the final quarter of incubation, an effective immunizing dose of live *Eimeria* sporocysts or oocysts, or a mixture thereof.
- 5 2. The method of claim 1 wherein the dose comprises  $10^2$  to  $10^8$  sporocysts or oocysts, or a mixture thereof wherein the total number of said sporocysts and oocysts ranges from  $10^2$  to  $10^8$ .
3. The method of claim 1 wherein the dose comprises  $10^2$  to  $10^5$  sporocysts or oocysts, or a mixture thereof wherein the total number of said  
10 sporocysts and oocysts ranges from  $10^2$  to  $10^5$ .
4. The method of claim 1 wherein the dose comprises  $10^5$  to  $10^7$  sporocysts or oocysts, or a mixture thereof wherein the total number of said sporocysts and oocysts ranges from  $10^5$  to  $10^7$ .
5. The method of claim 1 wherein the dose comprises  $10^4$  to  $10^8$   
15 sporocysts or oocysts, or a mixture thereof wherein the total number of said sporocysts and oocysts ranges from  $10^4$  to  $10^6$ .
6. The method of claim 1 wherein the dose comprises  $10^3$  to  $10^6$  sporocysts or oocysts, or a mixture thereof wherein the total number of said sporocysts and oocysts ranges from  $10^3$  to  $10^6$ .
- 20 7. The method of claim 2 wherein the domesticated bird is a chicken.
8. The method of claim 7 wherein the dose comprises sporocysts or oocysts, or a mixture thereof, of two or more species of *Eimeria* selected from the group consisting of *E. tenella*, *E. acervulina*, *E. maxima*, *E. necatrix*, *E. mitis*, *E. praecox*, and *E. brunetti*.
- 25 9. The method of claim 8 wherein the dose is administered by *in ovo* injection.
10. The method of claim 2 and further comprising administering *in ovo* an immune stimulant at any time during incubation.
11. The method of claim 10 wherein the immune stimulant is administered  
30 *in ovo* simultaneously with the dose of sporocysts or oocysts, or mixture of said sporocysts and oocysts.
12. The method of claim 2 wherein the dose comprises oocysts.
13. The method of claim 2 wherein the dose comprises sporocysts.

14. The method of claim 13 wherein the sporocysts have been purified to remove oocysts.
15. The method of claim 2 wherein the domesticated bird is a turkey.
16. The method of claim 15 wherein the dose comprises sporocysts or  
5 oocysts, or a mixture thereof, of two or more species of *Eimeria* selected from the group consisting of *E. meleagrititis*, *E. adenoeides*, *E. gallopavonis*, *E. dispersa*, *E. meleagridis*, *E. innocua*, and *E. subrotunda*.
17. The method of claim 16 wherein the dose is administered by *in ovo* injection.
- 10 18. The method of claim 2 wherein the domesticated bird is a game bird, duck or ratite.

# INTERNATIONAL SEARCH REPORT

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<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 6 A61K39/012		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 6 A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 291 173 (EMBEX INC.) 17 November 1988 cited in the application see page 2 - page 3 ---	1-18
A	THE JOURNAL OF PARASITOLOGY, vol. 56, no. 4, 1970 pages 656-662, T.K.JEFFERS ET AL. 'Embryonic Response to Eimeria Tenella Infection' cited in the application see abstract --- <div style="text-align: center;">-/--</div>	1-18
<div style="display: flex; justify-content: space-between;"> <span><input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.</span> <span><input checked="" type="checkbox"/> Patent family members are listed in annex.</span> </div>		
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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LES COLLOQUES DE L'INRA, vol. 49, 1989 pages 655-660, T.L.FREDERICKSEN ET AL. 'In Ovo administration of a potent recombinant coccidial antigen vaccine in poultry' see abstract ---	1-18
A	EP,A,0 650 733 (AMERICAN CYANAMID COMPANY) 3 May 1995 see page 2 - page 7 ---	1-18
A	WO,A,94 16725 (MERCK & CO, INC.) 4 August 1994 see page 1 - page 2 -----	1-18

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Application No

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-291173	17-11-88	CA-A- 1326818 JP-A- 1025731 ZA-A- 8802708	08-02-94 27-01-89 17-10-88
EP-A-650733	03-05-95	AU-B- 7763294 CA-A- 2134835 JP-A- 7206705	18-05-95 04-05-95 08-08-95
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